and a draft USP general chapter on CE has been published (*Pharm Forum* 1996) in anticipation of future monographs containing CE analytical methods.

Conclusions

The use of CE in the analysis of pharmaceuticals is now becoming firmly established as a useful complement and alternative to the more widely employed technique of HPLC. The major attractions of CE are that considerable cost and time savings are possible, especially in the areas of chiral analysis and in the determination of solutes having limited or no chromophore. The recent advent of the use of nonaqueous solvents in CE should extend further the application range of the technique. Routine CE methods have been established in many industrial laboratories and CE methods have been successfully submitted to regulatory authorities.

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Chiral Separations: Liquid Chromatography

W. J. Lough, University of Sunderland, Sunderland, UK

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Introduction

Between the mid-1980s and the mid-1990s great advances were made in the development of commercially-available methodologies for the separation of enantiomers. These developments were undoubtedly catalysed by the need to determine the enantiomer content of chiral drug substances, chiral drug products and samples of chiral drugs in biological fluids in the pharmaceutical research and development environment. It had long been recognized that, since it is possible to distinguish between enantiomers in a chiral environment, enantiomers might be expected to have different effects on the body which contains potential chiral 'selectors' such as proteins, peptides, carbohydrates and enzymes. The action of a drug on the body or of the body on the drug involves many processes including the following:

- active transport
- plasma binding
- tissue binding
- receptor protein binding
- drug metabolism
- active secretion.

These and many of the other processes involve or may involve mediation by a protein or enzyme so that there is no shortage of opportunities for chiral discrimination to take place. As a consequence it is actually quite rare for enantiomers to have very similar pharmacological and toxicological properties. They must therefore be treated as if they were different drugs, and their use in combination in a drug product in a fixed 50 : 50 ratio as the racemate must be justified to the governmental regulatory bodies which issue licences to companies to produce and market drugs. Not only is this usually not justifiable,

but also when it is justifiable the burden of justification might be such that it is even conceivable that it might be more convenient to proceed with a single enantiomer in any case.

The classic case of thalidomide, developed as a non-addictive alternative to barbiturates, is frequently cited as an illustration of the extreme case, where the desired pharmacological activity resides in one enantiomer and the other enantiomer has all the undesirable toxic effects. This is in fact an oversimplification of the true situation and the emphasis on this one case does nothing to allude to the wide range of other possible cases that might arise. There is no doubting though that the thalidomide case served not only to highlight the potential problems inherent in developing racemic drugs but also it triggered off the greater awareness of drug safety that led to the extensive regulatory frameworks that today control the production and sale of pharmaceuticals. Just as there was a time lag before there was a readily available capability to resolve enantiomers, so there was a time lag before single enantiomer chiral synthetic drugs were being brought onto the market.

Application Areas

Many of the naturally-occurring chiral drugs and chiral drugs derived from naturally-occurring subunits contain multiple chiral centres. Penicillin antibiotics and steroids are cases in point. For such compounds it would be exceptional for there to be a need to determine the enantiomer of the drug as an impurity as this would involve inversion of the stereochemistry at all chiral centres. What is more likely to be an impurity is an epimer where there is inversion at one of the chiral centres only. Since the chiral drug and one of its epimers are diastereomers they may be separated by high performance liquid chromatography (HPLC) on an achiral column.

For a different reason a chiral separation is also not usually required for the drug substance of a chiral drug marketed as the racemate, a 50 : 50 mixture of the enantiomers. If there is no chiral intervention in the synthesis there would be little reason to suspect that anything other than a 50 : 50 mixture was present. Accordingly in pharmacopoeial monographs it is usually sufficient to establish for the drug substance that e.g. the optical rotation lies between $+0.15^\circ$ and -0.15°. Chiral LC would only be required if 'chiral switch' were being contemplated. This might happen if it was thought that a significantly better drug could be had by substituting a single enantiomer for the racemate in cases when the racemic drug had been developed and licensed before the significance of chirality in drugs had been fully appreciated and before chiral resolution had become commonplace. The first step in the chiral switch decision making process, even before resolving, isolating and testing the individual enantiomers, would often be to study the fate of the individual enantiomers in the body following dosing with the racemate. It is therefore necessary to have a method capable of determining low levels of each enantiomer in biological fluids. To study the enantiomers in this way in the presence of the other enantiomer does not necessarily give a close approximation to the pharmacokinetics of the enantiomers when they are dosed on their own, but it does give an early indication as to whether there is a major difference in the way each enantiomer interacts with the body.

The main area in which enantiospecific LC methods are called for is in the research, development and production of synthetic single enantiomer drugs. This category mainly consists of drugs which arise directly from the selection of the most suitable enantiomer of a new chemical entity but will also include those which arise from a chiral switch. The actual applications of chiral LC which are important and most commonly carried out are described below.

Preparative Resolution of Individual Enantiomers

Clearly the individual enantiomers need to be isolated before they can be tested for their pharmacological action. This is very often done by semi-preparative LC since often only milligram quantities are needed. Preparative even up to Kg scale is an option for the actual production of a drug substance but most often a stereospecific synthetic route is more commercially viable.

Trace Enantiomer Determination in a Drug Substance

The trace enantiomer must be treated like any other related substance and therefore must be quantified down to levels of 0.1% w/w. This is usually carried out as a separate exercise from the determination of other related substances.

Chiral Drug Bioanalysis

As already indicated, a chiral method for the determination of enantiomers plays an important role in the decision on which enantiomer should be developed as a drug candidate. For a new chemical entity this will take place in the discovery phase of pharmaceutical research and development but a few studies might still be needed in early development to ensure that there is no interconversion between enantiomers taking place in the body.

There are a few other instances where a chiral assay might be needed but, for example, the determination of trace enantiomer in a formulated product would only be needed if it were known that it was a potential degradant.

Method Development

Now that the current scenario involves a strong preference for the development of single enantiomer drugs, the types of chiral analytical methods that are required has changed. However the first step in method development is as always to achieve chiral resolution. This has never been a matter of trial and error. By the late 1980s there was already a large number of chiral stationary phases (CSP) available for direct resolution by chiral LC. Despite this it was possible to group these together in classes by their mode of action and from this deduce which types of enantiomeric drugs might be separable using each class. For example, the acidic drug ibuprofen is highly protein bound in plasma and it is therefore no surprise that its enantiomers may be separated on a column containing an albumin CSP since albumin is the major binding plasma protein for acidic drugs. As CSP became more effective and more sophisticated by incorporating multiple modes of interaction, such simple predictions were no longer possible.

Fortunately there were 'intelligent' approaches that could be used to address this paradoxical situation. These are outlined below.

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NMR Modelling

If splitting of the signals for the analyte is observed when its ^{1}H NMR spectrum in the presence of the chiral selector that is used in the chiral HPLC column is recorded, then if the solvent used in ^{1}H NMR is the same or similar to that which might be used in HPLC then it might be expected that chiral resolution will be observed in HPLC.

Molecular Modelling

The molecular orbitals involved in the transient diastereomeric complexes formed between the individual enantiomers and the chiral selector determine whether there is a large enough energy difference for the selector to be able to distinguish between the enantiomers at ambient temperature. While it is one thing to simulate selector approaching select and in a vacuum, it is an altogether more difficult matter to construct a meaningful simulation that incorporates mobile phase molecules, neighbouring chiral 'strands' on a CSP and the supporting stationary phase.

Databases

The most well known of these is *Chirbase*. Whether or not a separation will take place may be predicted by comparing with information on similar selector–selectand situations held on the database. This approach is reliant on the quality of the information held on the database and suffers from the weakness that it might be a subtle difference between the chiral drug being studied and a similar one described on the database that might be responsible for a quite marked difference in behaviour towards the chiral selector being considered.

Expert Systems

These systems are more versatile than databases in that they make decisions and learn from experience with each problem encountered. Again, each new problem might be subtly different from the one that has gone before and it might be this subtle difference that is critical with respect to which is the best chiral selector to use.

While these approaches have remained popular in academic circles, screening approaches have been more widely adopted in pharmaceutical research and development. This might typically involve the LC of the racemate being studied on three or four different chiral columns in a column-switching manifold, using a range of different mobile phases. Ideally in one overnight run the racemate would be analysed on each column using each of the mobile phases. The conditions which had shown the best resolution would then be identified by the computer controlling the system in order that they be further refined, if necessary, using mobile phase optimization so that by the morning the chiral method development would be complete. The increasingly widespread use of such screens may be attributed to the fact that the CSP used in them are now more effective to the extent that the screens are usually highly successful, with 'hit rates' for chiral resolution in the range 80–100% being quite common. Their acceptance in drug development in particular arises also because of the availability of highly automated instrumentation and the fact that in this environment, the purchase of a range of chiral LC columns which would generally be regarded as expensive is cost effective in the context of the benefit derived and the overall costs involved in pharmaceutical research and development. Modelling and database approaches have been overtaken because now it is just as quick to do the actual experiments.

Derivatized cellulose CSP with organic mobile phases usually play an important role in such screens. For example the use of ChiralCel OD, ChiralPak

AD, ChiralPak AS and the Whelk-O CSP with *n*-hexane-propan-2-ol (85 : 15, v/v) containing 0.5% triethylamine and *n*-hexane-propan-2-ol (85 : 15, v/v) containing 0.5% trifluoroacetic acid as the mobile phase is highly effective. Remarkable though the success rate of such a screen might be it would be the complete answer to all chiral method development problems, since it would not be ideal for giving optimum conditions for very polar compounds and would not be suitable in practice for samples presented as salts or as aqueous solutions. For these cases a screen using a cyclodextrin CSP and different macrocyclic antibiotic CSP with polar organic and aqueous–organic mobile phases would likely be more appropriate. Such a screen is often as effective as the type involving organic mobile phases and, for the polar organic mobile phase in particular, the final optimization can be quite simple.

Of course, achieving the chiral separation is not the end of the story. There are additional difficulties peculiar to each type of application.

Preparative Resolution of Individual Enantiomers

An additional difficulty in preparative resolution is the issue of time and money. Therefore as high as possible a sample load will be applied to the column. This leads to loss of efficiency and subsequent loss of resolution. Although loss of baseline resolution can be compensated for by collecting or 'shaving off' a leading part of the first peak and the second part of the second peak with the portion collected where the peaks overlap being recycled later, it is a general rule that it is better to start off with very high chiral resolution before commencing with scale-up.

The range of approaches to preparative chiral LC is dealt with elsewhere in this Encyclopedia. However the approach of choice will depend on the circumstances. In the 'drug discovery' phase of pharmaceutical research and development it will often be sufficient to obtain mg-quantities of each enantiomer for pharmacological testing or perhaps g-quantities if preliminary information on pharmacokinetics or toxicology is required before proceeding to early 'development'. Under these circumstances chemists are usually prepared to put up with the cost of using a semi-preparative ($\sim$7 mm i.d.) or preparative (16–22 mm i.d.) derivatized polysaccharide column, as this works out more economical than taking the time that might be needed to develop a 'cheaper' method.

The economics in a Pilot Plant or Production environment are entirely different. Conventional preparative chiral LC on scaled-up versions of analytical columns would be too expensive in terms not only of money but also time (which of course is money!). In this scenario the simulated moving-bed approach to preparative work, in which the mobile phase is cycled round a closed system of connected preparative chiral columns and resolved enantiomers are periodically drawn from the system at set points, is becoming increasingly popular. However, old habits die hard and if organic chemists cannot devise a stereoselective synthesis they may then still revert to a fractional crystallization to isolate the large quantities of one enantiomer that are required.

Trace Enantiomer Determination in Drug Substance

As for preparative chiral LC, there are additional problems that need to be dealt with over and above simply achieving chiral resolution. Generally it will be necessary to be able to determine down to 0.1% of the trace unwanted enantiomer in the bulk drug substance. Since peaks in HPLC are more often than not slightly asymmetric with a degree of peak tailing, this determination of enantiomeric impurity is more difficult when the impurity peak elutes after the main peak. As with the problem with scale-up in preparative chiral LC, one approach is to attempt to obtain a separation that is significantly better than baseline resolution. In this way the impurity peak is well clear of the tail of the main peak with the result that its area may be more accurately integrated. An alternative approach is to use the chiral stationary phase or chiral mobile phase based on the antepode of the chiral selector enantiomer used initially. Under these circumstances the enantiomeric impurity peak will elute first and will be much easier to determine. Switching the chirality of the selector in this way may be carried out for small molecule chiral selectors such as ligand-exchange, synthetic multiple interaction or Pirkle-type, ion-pair and crown ether selectors. However it is a weakness of the broader spectrum chiral selectors based on larger molecules, e.g. proteins, cyclodextrins, derivatized polysaccharides and macrocyclic antibiotics, that such a simple reversal of retention order is not possible.

Another difficulty is that the enantiomeric impurity must also be resolved from other structurally-related impurities. In general this is possible but it is more difficult to separate all structurally-related impurities from one another with a method using a chiral stationary phase or mobile phase additive. It is for this reason, and the fact that it is not prudent to use expensive chiral stationary phases more than is absolutely necessary, that the determination of trace enantiomeric impurity is almost always performed as a separate test.

Chiral Drug Bioanalysis

The difficulties over and above that of achieving a chiral separation are much more apparent for chiral drug bioanalysis than for the other two application areas that have been discussed. In any determination of drugs in biological fluids, problems arise because the levels of drug are invariably low and the matrix is invariably complex. Often also, for example in clinical trials to assess the safety and efficacy of a drug on patients, sample numbers may be very high. However this latter issue does not normally apply to chiral drug bioanalysis since, as has already been indicated, a method which is not stereoselective may be used if it can be shown in early development that the drug does not racemize or invert to its opposite enantiomer when in the body. Accordingly, some of the complex, automated methods involving column switching mooted in the early days of chiral drug bioanalysis when there were still many racemic drugs in the development phase are no longer appropriate. What is needed is simple methods that don't take too long to develop. As it is not sensible to load 'dirty' samples from biological fluids onto expensive chiral columns, the most common approach is to use extensive sample pre-treatment. This would involve concentration as well as clean-up so that the levels of drug loaded onto the column would be well above the limit of quantitation.

While the use of chemical derivatization is another approach that may be adopted to ensure that the chiral drug and its enantiomer, if present, are easily detected, chiral derivatization followed by achiral LC is an approach that needs to be used with caution. There is now greater awareness of potential difficulties such as racemization during the derivatization reaction, kinetic resolution caused by different reaction rates for the enantiomers and differing detector responses for the diastereomeric products. Apart from the racemization issue and the fact that an extra step is involved in the analytical procedure, there are no such drawbacks when achiral derivatization is used followed by chiral LC. Further, when the derivatizing agent contains a π-electron rich aromatic ring system and the chiral stationary phase used is a synthetic multiple interaction or Pirkle-type containing a π-electron deficient aromatic ring system in the chiral selector, then very good chiral resolution as well as easy detectability may be anticipated.

A more recent approach to chiral drug bioanalysis that fulfils all the requirements is the use of solid phase extraction (SPE) followed by direct injection of a large volume of the SPE eluate onto a microbore LC containing the porous graphitic packing material Hypercarb^{R} with a mobile phase containing a chiral mobile phase additive. Low limits of quantitation are ensured by the focussing effect that takes place because the eluate from a C-18 or phenyl-SPE cartridge is les strongly eluting than the mobile phase in the microbore LC column. Hypercarb^{R} is used because it is both highly retentive, thereby requiring a mobile phase that is more strongly eluting than that required to elute the analyte from the C-18 or phenyl-SPE cartridge, and is a very effective achiral support for use in methods that use a chiral mobile phase additive. The latter property is also at least in part due to its high retentivity.

Future Perspectives

Chiral LC of drugs is one of the scientific success stories of the late 20th century. It is now a mature area of research even although it only really began in the 1980s. While it would be useful to have more economical preparative chiral LC methods and to be able to determine enantiomeric and other structurally-related impurities simultaneously, it has to be said that these are not critical needs and that the major advances have almost certainly already taken place.

See also: **III / Chiral Separations:** Chiral Derivatization; Liquid Chromatography.

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Chromatographic Separations

J. Vessman, AstraZeneca R&D Mölndal, Mölndal, Sweden

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Overview

Pharmaceutical analysis is traditionally defined as analytical chemistry dealing with drugs both as bulk drug substances and as pharmaceutical products (formulations). However, in academia, as well as in the pharmaceutical industry, other branches of analytical chemistry are also involved, viz. bioanalytical chemistry, drug metabolism studies and analytical biotechnology. The development of drugs in the pharmaceutical industry is a long-term process, often taking more than a decade from the start of a research project to the appearance of a drug on the market. That process involves several decision points, such as the choice of the candidate drug after the preclinical screening phase, the investigational new drug (IND) application before testing the compound for the first time in man, and finally the new drug application (NDA) which summarizes the data obtained from all the studies needed for marketing approval of the drug as a medicine. In all these steps, especially the IND and NDA, the amount of data generated is enormous. Analytical chemists take part in many of the studies that constitute this documentation. Substance quality and its specifications are based on substance analysis, and that knowledge is later used for quality control during full-scale production. Product analysis involves dealing with the various formulations and starts after the IND has been approved. The results from such work lead to specifications that form the basis for the quality control of the product. For both substances and formulations there is an increasing interest in the introduction of process analytical chemistry.

Biomolecules, i.e. macromolecules such as proteins or hormones, either produced by isolation from biological sources or by means of biotechnology, must also be subjected to careful analytical control. Thus whilst the analytical tasks required for biomolecules are somewhat different from those of ordinary pharmaceuticals when it comes to regulation and documentation of their quality and properties they definitely belong to the same group.

There are a number of regulations that have to be followed in the development of pharmaceuticals as well as in their production. Regulatory approval is required prior to the IND and before marketing is licensed (NDA). Today clinical trials also undergo scrutiny by the authorities.

An important part of the development process is safety evaluation, primarily the toxicology tests, which run from 6 to 24 months in different species. During this time bioanalytical studies are performed as well as control of the formulations used in the tests. After approval for marketing, the authorities exercise control of products on the market and require post production stability data. Public interest in the quality of drugs is also reflected in the compilation of substance monographs in compendia that are known as pharmacopoeias. In addition to collections of substance monographs these pharmacopoeias contain general analytical methods and some also contain monographic requirements on the formulation of the substances.

This article provides an overview of mainly substance and product analysis (traditional pharmaceutical analysis), as used in the pharmaceutical industry. The support of other branches of analytical chemistry will be mentioned.

Bulk Drug and Pharmaceutical Products

Common Features

Identity testing Identity testing is used to verify that the drug substance is what it is stated to be or that the formulation contains the correct compounds. Infrared (IR) spectra are used quite extensively in industry, whereas the pharmacopoeias often have a set of alternative tests. These can be colour reactions, melting point of a compound or derivative, optical